

## **CERTIFICATION REPORT**

**Certification of reference materials of soya seed powder  
with different mass fractions of genetically modified  
Roundup Ready<sup>®</sup> soya**

**Certified Reference Materials ERM<sup>®</sup>-BF410k**  
(ERM<sup>®</sup>-BF410ak, ERM<sup>®</sup>-BF410bk,  
ERM<sup>®</sup>-BF410dk, ERM<sup>®</sup>-BF410gk)

The mission of the IRMM is to promote a common and reliable European measurement system in support of EU policies.

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ERM<sup>®</sup>-BF410dk, ERM<sup>®</sup>-BF410gk)

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## GLOSSARY

<i>aat</i>	aspartate aminotransferase gene
ANOVA	analysis of variance
<i>b</i>	slope in the equation of linear regression $y = a + bx$
CaMV	cauliflower mosaic virus
CP4	<i>Agrobacterium tumefaciens</i> strain from which the enzyme 5-enolpyruvylshikimate-3-phosphate synthase gene was extracted
CRM	Certified Reference Material
<i>Ct</i> -value	number of PCR cycles to pass a set cycle threshold
$df_{wb}$	degrees of freedom within bottle
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
<i>epsps</i>	5-enolpyruvylshikimate-3-phosphate synthase gene
ERM <sup>®</sup>	European Reference Material
FAM	6-carboxy fluorescein
GM	genetically modified
GMO	genetically modified organism
IRMM	Institute for Reference Materials and Measurements
<i>k</i>	coverage factor
KFT	Karl Fischer titration
LOD	limit of detection
MGBNFAQ	Minor groove binding non fluorescent quencher
$MS_{bb}$	mean sum of squares between bottles
$MS_{wb}$	mean sum of squares within bottle
<i>N</i>	number of samples analysed
<i>n</i>	number of subsamples analysed
n.a.	not applicable
NIB	National Institute of Biology
NOS	terminator sequence derived from the nopaline synthase gene from the <i>Agrobacterium tumefaciens</i> Ti plasmid
PCR	polymerase chain reaction
PSA	particle size analysis by laser diffraction
rt-PCR	real-time PCR
RUR soya	Roundup Ready <sup>®</sup> soya ( <i>Glycine max</i> L.) event GTS 40-3-2 containing the glyphosate herbicide resistance caused by the expression of the enzyme 5-enolpyruvylshikimate-3-phosphate synthase isolated from <i>Agrobacterium tumefaciens</i> strain CP4
<i>s</i>	standard deviation
$s_{bb}$	standard deviation between vials
$s_{bb, rel}$	relative $s_{bb}$
SI	International System of Units
TAMRA	6-carboxy-tetra-methyl-rhodamine
TaqMan <sup>®</sup>	<i>Thermus aquaticus</i> (Taq) DNA polymerase-based technology for fluorescent signal generation during rt-PCR
<i>u</i>	standard uncertainty
<i>U</i>	expanded uncertainty
$u^*_{bb}$	standard uncertainty related to the between-vial heterogeneity that can be hidden by the method repeatability
$u^*_{bb, rel}$	relative $u^*_{bb}$
$u_{bb}$	standard uncertainty related to the between-vial heterogeneity
$u_{char}$	standard uncertainty related to the characterisation
$u_{lts}$	standard uncertainty related to the long-term stability of the material
$u_{lts, rel}$	relative $u_{lts}$
$\bar{X}$	average
35S	35S promoter sequence derived from the cauliflower mosaic virus

## 1 Introduction and design of the project

Legislation in the European Union demands the labelling of food and feed products consisting of or containing "more than 0.9 % genetically modified organisms" (GMOs) [1]. This is the labelling threshold level for GMOs that are authorised in accordance with Community legislation. In general, this demands on the one hand, the development and validation of reliable GMO quantification methods, and on the other hand the production of reference materials for the quality control and calibration of these procedures.

Monsanto Company (St. Louis, MO, USA) has developed the genetically modified (GM) soya event GTS 40-3-2, which is commonly referred to as Roundup Ready<sup>®</sup> soya, due to its resistance towards the herbicide Roundup<sup>®</sup>. Following Commission Regulation (EC) No 65/2004 [2], the GTS 40-3-2 soya received the unique identifier code MON-Ø4Ø32-6. The genetic modification codes for a glyphosate herbicide resistance caused by the expression of the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) isolated from the common soil bacterium, *Agrobacterium tumefaciens* strain CP4.

In 2001 the Institute for Reference Materials and Measurements (IRMM, Geel, BE) and Monsanto agreed on the development and production of a reference material for the quantification of GTS 40-3-2 soya. As the previous certified reference material (CRM) ERM<sup>®</sup>-BF410 [3] in 2007 was closely to be sold out, it was decided to produce a new series of Roundup Ready (RUR) soya reference material. This new series received the code ERM-BF410k with 'k' indicating the replacement batch. For the production of the CRMs containing different mass fractions of the genetically modified RUR soya, GM seeds and non-GM seeds were used.

## **2 List of participants**

### **Processing, characterisation and stability studies**

European Commission, Joint Research Centre, Institute for Reference Materials and Measurements (IRMM), Geel (BE), accred. ISO Guide 34 and ISO/IEC 17025 BELAC 268-TEST.

### **Homogeneity studies**

National Institute of Biology (NIB), Ljubljana (SL), ISO/IEC 17025 Slovenska akreditacija št./no. LP-028.



### 3 CRM processing

#### 3.1 Characterisation of the base materials

For the preparation of the CRMs, Monsanto Company supplied non-modified soya seeds and modified soya seeds to IRMM. The Roundup Ready (RUR) soya seeds are homozygous GM seeds of the variety AG5602. The non-GM comparator variety is A1900. The seeds used for the production of ERM-BF410k are the same varieties of the same origin as the ones used to produce ERM-BF410, stored at our premises. Therefore characterisation measurements of the base material were not repeated, but taken from the ERM-BF410 certification [3].

The delivered non-GM seed batches have been tested at Monsanto for their purity using an enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR) for further confirmation of the genetic identity. The company delivered the following informations:

- sixty pools each of 50 seeds of the conventional non-GM soya variety A1900 were qualitatively tested by immunoassay for the CP4 EPSPS protein and all were found negative.
- additionally, RUR soya GTS 40-3-2 event-specific PCR was performed on the non-GM status of soya variety A1900. For the PCR methods aspartate aminotransferase gene (*aat*) was used as control. For the A1900 seeds, three pools of 50 seeds tested negative by PCR for GTS 40-3-2 and positive for the *aat* gene. These PCR results confirmed the immunoassay results on a genetic level (Table 1).

The reported purity and genetic composition of both soya seed batches were verified at IRMM in the frame of the previous certification of ERM-BF410 [3].

For the random check of Monsanto's statement on the purity and the uniform zygoty of the GM RUR soya seeds, 50 randomly chosen seeds were germinated on moistened paper in a growing chamber during 15 days. 50 mg pieces from young leaves were sampled from 46 plants (4 plants did not germinate). The plant tissues were disrupted in a lysis buffer using the Mixer Mill MM 300 (Retsch, Haan, DE) and 3-mm tungsten carbide beads (Qiagen, Hilden, DE). Total DNA was extracted using the DNeasy® 96 Plant kit (Qiagen, Hilden, DE). The total DNA was analysed on agarose gel and quantified using the PicoGreen® dsDNA quantitation kit (Molecular Probes Europe, Leiden, NL). The detection of RUR soya events was performed following the TaqMan® Universal PCR Master Mix protocol (Applied Biosystems, Foster City, CA, USA). A primer pair specific for the modified *epsps* gene (the *Petunia hybrida* 5-enolpyruvylshikimate-3-phosphate synthase chloroplast transit protein (CTP) sequence was fused to the N-terminus of the CP4 *epsps* gene to deliver the CP4 EPSPS protein to the chloroplast, the site of EPSPS activity and glyphosate action) was used together with a reporter-dye labelled with FAM. For calibration, RUR soya DNA extracted from GM seeds diluted in non-GM DNA extracted from non-GM seeds was used.

The calculated percentage of GMO was then corrected using primers targeting the soybean lectin gene as an additional estimation of total soybean DNA. The calculated average percentage of RUR soybeans was  $105.51 \% \pm 11.40$  ( $n = 46$ ) and no samples had a percentage value below 87.3 %. It could be concluded, that all plants were therefore homozygous (Table 1).

**Table 1: Genetic purity of the non-GM and GM seed batches used for the processing of ERM-BF410k with respect to RUR soya GM event GTS 40-3-2**

Batch	Test results reported by	Method applied	Number of seeds tested	Number of GM positive	Number of GM negative
Non-GMO	Monsanto	ELISA targeting the CP4 EPSPS protein	3000 <sup>1)</sup>	0	3000 <sup>1)</sup>
	Monsanto	event specific GTS 40-3-2 PCR <sup>3)</sup> [a]	150 <sup>2)</sup>	0	150 <sup>2)</sup>
GMO	IRMM	Epsps specific PCR <sup>3)</sup> [b]	46	46	0

<sup>1)</sup> Sixty pools of each 50 seeds were ground and tested negative for the CP4 EPSPS protein.

<sup>2)</sup> Three pools each of 50 seeds were ground and tested negative for event-specific PCR.

<sup>3)</sup> The used primer and probe sequences can be found in Annex I.

### 3.2 Processing of the base materials

The GM and non-GM base materials were processed separately. Cross-contamination and contamination with foreign DNA were avoided using glove box systems and clean laboratory clothing. All contact surfaces were treated with a DNA degrading solution prior to exposure to the base materials. An in-house validation study had proven beforehand that the solution degraded DNA effectively under the given conditions.

The soya seeds were rinsed in demineralised water, drained, and dried under vacuum at 30 °C. This resulted in a water mass fraction loss between 30 g/kg and 48 g/kg for both seed batches. The dried seeds were then milled using a high impact mill with a triangular ribbed open grinding track in order to obtain the ground base material. The high impact mill was flushed with nitrogen gas throughout the milling process and milling was interrupted if the temperature rose above 40 °C. An additional vacuum drying at 30 °C was carried out to further reduce the water content of the once ground base material. Each ground base material was mixed in a turbula mixer for 30 min to improve equal distribution of the different parts of the soya tissues separated by the milling process. The final water mass fraction in the non-GM and GM base materials was 11.7 g/kg and 6.1 g/kg respectively ( $n = 3$ ; measured by volumetric KFT). The average particle size of the non-GM and GM powder was determined in triplicate measurements by laser diffraction patterns (PSA, Sympatec, Clausthal-Zellerfeld, DE). The average particle size, calculated by the PSA software, was 112 µm for the non-GM powder and 178 µm for the GM powder. The powders were then stored in closed plastic containers.

### 3.3 Gravimetric preparation of GM mixtures

The ground base materials were used to produce a GM blank material and three powder mixtures containing mass fractions of RUR soya seed powder in non-GM soya seed powder at nominal levels of 1, 10 and 100 g/kg. All four materials, including the blank powder, were treated according to the same procedure. The powder materials were weighed using a calibrated balance with a relative standard uncertainty lower than 0.1 %. The masses of non-GM and GM soya powders required to produce the mixtures were corrected for the water mass fraction of the starting materials (Table 2). The starting materials were combined in one container, turbula-mixed for 30 min, and further mixed in a special dry-mixing device for another 2 min. The blank material was processed first, followed by the mixtures. The nominal mass fraction of 100 g/kg (ERM-BF410gk) was produced by mixing pure GM with pure non-GM ground base materials. The 10 g/kg (ERM-BF410dk) mass fraction was produced by further dilution of the 100 g/kg GM powder with pure non-GM powder, and the 1 g/kg (ERM-BF410bk) was likewise produced by further diluting the 10 g/kg mixture with non-GM powder.

In Table 2, the data supporting the calculation of the certified values are summarised.

**Table 2: Results from stepwise dilutions of ERM-BF410k based on gravimetry**

ERM	GM powder			Non-GM powder <sup>1)</sup>	Resulting GM mass fraction  [g/kg]
	Mass fraction [g/kg]	Water mass fraction [g/kg]	Mass [g]	Mass [g]	
BF410bk	10.00	11.0	399.80	3600.20	1.00
BF410dk	100.00	10.8	399.60	3600.20	10.00
BF410gk	1000	6.1	398.00	3602.00	100.00



<sup>1)</sup> The non-GM powder used for the gravimetric preparations of the CRMs had a water mass fraction of 11.7 g/kg and was considered to be free of 40-3-2 soya.

### 3.4 Additional milling of the powders

Due to the remaining presence of some coarse particles in the once-milled powders, an additional milling was done for improved DNA extractability. This second milling was done after the dry-mixing of the GM and non-GM base materials (Section 3.3) because experience showed that the powders resulting from a double milling are rather sticky and hence more difficult to mix during the preparation of the gravimetrical mixtures. For this reason, the coarse particles were first sieved out of the blank and the mixed powders, milled as before (Section 3.2), and then added back to the original main batch. Sieving was done in an industrial sieving machine using a sieve with a mesh of 500 µm, starting with the non-GM powder and followed by the nominal 1, 10 and 100 g/kg powders. The twice milled fraction corresponded to approximately 11 % of the mass of each of the powders. The main fraction with particles < 500 µm and the twice ground fraction were merged by a further turbula-mixing during 30 min to ensure the homogeneity of the final powder mixtures.

### 3.5 Bottling

The powders were filled in 10 mL brown glass vials using an automatic filling device. The first 30 vials of each batch were discarded as an additional precaution against carry-over contamination. Rubber stoppers were automatically placed in the neck of the vial. Before closure of the vials, air was evacuated in a freeze-drier and replaced by argon. The vials were closed inside the freeze-drier with the help of a hydraulic device and then sealed with aluminium caps to prevent accidental opening during storage and transport. Colour-coded caps were used for easy identification of the different GM levels: nominal mass fraction 0 g/kg = silver, nominal 1 g/kg = yellow, nominal 10 g/kg = red, nominal 100 g/kg = brown, consistent with the cap colours of previous IRMM CRMs. Each of the vials was identified by a numbered label indicating the ERM code specific for each concentration level (Figure 1). Following the inventory control and the selection of vials for future analysis according to a random stratified sampling scheme, the vials were brought to a storage room for long-term storage in the dark at 4 °C.

<p><b>ERM-BF410ak</b> Sample 000</p>  <p><b>Certified Reference Material</b> <b>Roundup Ready® Soya (blank)</b> For laboratory use only, not for drugs, household or other use</p> <p>European Commission, JRC, IRMM Retieseweg 111, 2440 Geel, Belgium Tel: +32 (0)14 571 722; Fax: +32 (0)14 590 406</p>	<p><b>ERM-BF410bk</b> Sample 000</p>  <p><b>Certified Reference Material</b> <b>Roundup Ready® Soya</b> For laboratory use only, not for drugs, household or other use</p> <p>European Commission, JRC, IRMM Retieseweg 111, 2440 Geel, Belgium Tel: +32 (0)14 571 722; Fax: +32 (0)14 590 406</p>
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<p><b>ERM-BF410dk</b> Sample 000</p> <p>Certified Reference Material <b>Roundup Ready® Soya</b> For laboratory use only, not for drugs, household or other use</p> <p>European Commission, JRC, IRMM Retieseweg 111, 2440 Geel, Belgium Tel: +32 (0)14 571 722; Fax: +32 (0)14 590 406</p>	<p><b>ERM-BF410gk</b> Sample 000</p> <p>Certified Reference Material <b>Roundup Ready® Soya</b> For laboratory use only, not for drugs, household or other use</p> <p>European Commission, JRC, IRMM Retieseweg 111, 2440 Geel, Belgium Tel: +32 (0)14 571 722; Fax: +32 (0)14 590 406</p>
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**Figure 1: Prototype labels for the ERM-BF410k series. ERM-BF410ak, ERM-BF410bk, ERM-B410dk and ERM-BF410gk refer to the nominal mass fraction 0 g/kg, 1g/kg, 10 g/kg and 100 g/kg RUR soya, respectively.**

### 3.6 Processing control

The residual mass fraction of water in the final CRMs was determined by volumetric KFT in randomly selected vials from each of the powder mixtures (Table 3). As a result of the drying steps during the processing, the water mass fractions in the final CRM powders were quite low.

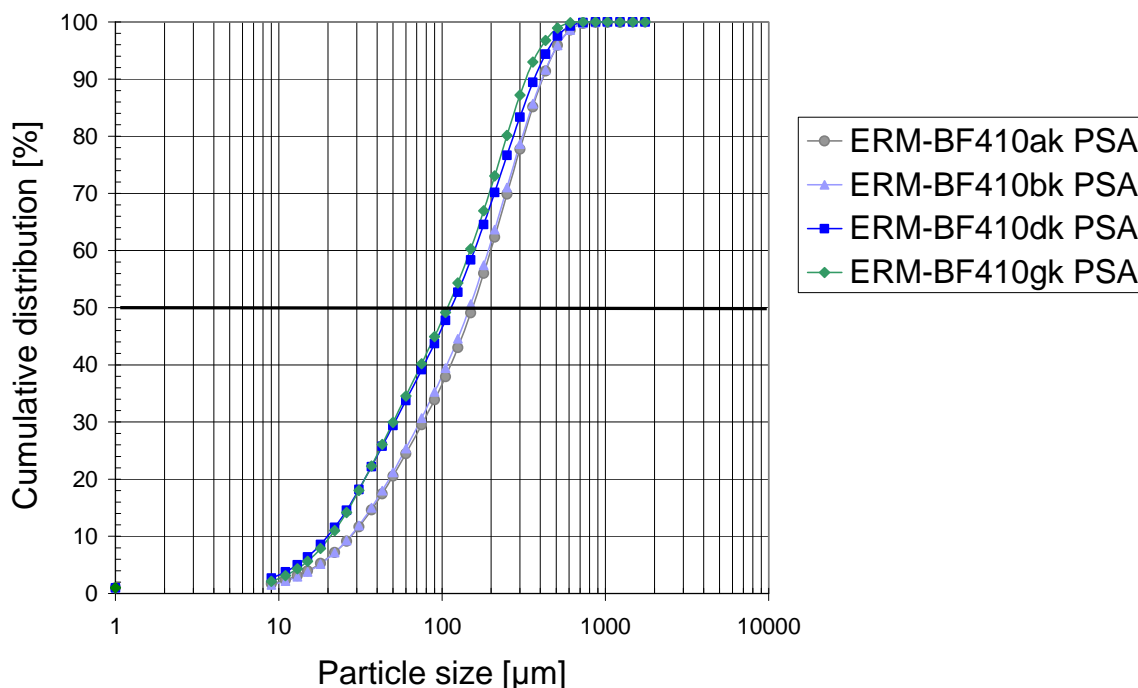
The hygroscopic behaviour of ERM-BF410k was investigated using 2 units of ERM-BF410ak and 2 units of ERM-BF410gk. For ERM-BF410ak a water uptake of 15.7 g/kg and 37.5 g/kg following 1 h incubation at 43 % and 75 % relative humidity, respectively, was recorded. For ERM-BF410gk a comparable water uptake of 16.1 g/kg and 30.6 g/kg was found. Similar results were found in the past for CRMs processed in the same way. As a result of the strong hygroscopicity of the powders, it is recommended to close the vials immediately after taking a subsample from the vial.

**Table 3: Water mass fraction in CRM ERM-BF410k determined by volumetric KFT ( $N = 10$ ,  $n = 1$ )**

CRM	Water mass fraction [g/kg]	
	$\bar{x}$	$s$
ERM-BF410ak	5.1	1.1
ERM-BF410bk	9.8	1.9
ERM-BF410dk	15.0	2.8
ERM-BF410gk	15.3	2.1

Five randomly selected vials from each of the powder mixtures were analysed for their particle size distribution based on laser diffraction patterns (PSA, Sympatec, Clausthal-Zellerfeld, DE). From each vial, 3 subsamples were analysed. Note that the data generated by this analysis are based on the equivalent volume diameter fraction (see legend of Figure 2). The powders had a similar particle size distribution profiles. The average particle size, calculated by the PSA software, was 192  $\mu\text{m}$ , 187  $\mu\text{m}$ , 159  $\mu\text{m}$  and 146  $\mu\text{m}$  for ERM-BF410ak, bk, dk and gk respectively ( $\bar{x} \pm s$  for all powders = 171  $\pm$  22  $\mu\text{m}$ ) and a number fraction of 99.98 % of particles had a size below 1030  $\mu\text{m}$ . Visual inspection of the powders, however, showed that some coarser particles are present in the vials, which may result from particle aggregation or from insufficient milling. Their rare occurrence should, however, not affect the certified value or the use of the CRMs. The average particle size of 171  $\mu\text{m}$  was used for the calculation of the minimum sample intake for the four CRMs (Section 4.2).

It is concluded from the results of the particle size analysis method that the powders are sufficiently fine for an adequate extraction of genomic DNA [6].



**Figure 2: Average particle size distribution in ERM-BF410k by PSA ( $N = 5$ ,  $n = 3$ ). The cumulative distribution of particles derived from laser light scattering data (PSA) is based on their equivalent volume diameter, i.e. the maximum diameter of the particles derived from the volume occupied upon rotation of the particles.**

Three of the described CRMs are mixtures of GM and non-GM soya powders, produced gravimetrically and certified for their GM powder mass fraction. Quantification of the GM content is, however, based on rt-PCR, which measures DNA copy numbers. The mass fraction of DNA in both base materials was determined by a slight modification of the classical fractionation method developed by Ogur and Rosen [7]. Following the sequential removal of ethanol-, ethanol-ether- and acid-soluble compounds, the DNA was obtained by repeated acidic extraction with 0.84 mol/L perchloric acid at 70 °C. The extractable mass of DNA in 100 mg soya powder was measured spectrophotometrically after derivatisation with diphenylamine, which reacts specifically with 2-deoxyriboses linked to purine nucleobases [7, 8]. The results are shown in Table 4. To reveal a difference between both powders, the ratio of their extractable DNA content was calculated as follows:

$$\frac{\text{Extractable mass of DNA in 100 mg RUR soya powder}}{\text{Extractable mass of DNA in 100 mg non - GM soya powder}}$$

The ratio of the DNA mass extractable from 100 mg of GM and non-GM soya powder was found to be  $1.18 \pm 0.08$  ( $N = 9$ ). A  $t$ -test confirmed the existence of a significant difference between the DNA mass extracted from the GM and non-GM powder by the modified Ogur and Rosen [7] method (95 % confidence level). There is therefore evidence that the DNA mass present in the two powders differ. Consequently the GM mass fractions of ERM-BF410k prepared by gravimetry are not identical to the GM copy number measured by rt-PCR.

For the in-house verification of the non-GM seeds the ground base material was analysed by three different real-time PCR (rt-PCR) methods. Two screening methods, one targeting the 35S sequence originating from the cauliflower mosaic virus (CaMV) and one targeting the 3' untranslated region of the nopaline synthetase gene (NOS) sequence originating from *Agrobacterium tumefaciens*, and a PCR method targeting the 5-enolpyruvylshikimate-3-

phosphate synthase gene (*epsps*) were used. The values were below the LOD of the used methods (Table 4). The LOD was calculated as  $(3.3 \cdot s)/b$ , with  $s$  representing the standard deviation of the lowest GM mass fraction and  $b$  the slope of the calibration curve. The efficiency of the amplification was determined, based on the slope of the regression line between the GM mass fraction and the  $C_t$ -values, which should be around the theoretical value of 3.322.

**Table 4: Quantification of potential GM contaminations in the non-GM base material using 35S, NOS and *Epsps* specific real-time PCR. From five individual samples, three extractions using 20 mg subsample were analysed in triplicate ( $N = 5$ ,  $n = 3$ ) [b, c, d]**

Method applied <sup>1)</sup>	Mass fraction [g/kg]	$U (k = 2)$ [g/kg]
35S PCR [c]	< 1.2 <sup>2)</sup>	-
NOS PCR [d]	< 0.9 <sup>3)</sup>	-
<i>Epsps</i> specific PCR [b]	< 0.2 <sup>4)</sup>	-

<sup>1)</sup> Primers used can be found in the Annex I.

<sup>2)</sup> The value was below the LOD of the method (1.2 g/kg).

<sup>3)</sup> The value was below the LOD of the method (0.9 g/kg).

<sup>4)</sup> The value was below the LOD of the method (0.2 g/kg).

Until the production of ERM-BF410k was started, the soya seeds were stored at -20 °C in the dark. Twenty-two kg of non-GM soya seeds and 10 kg of RUR soya seeds were used for the processing described in the following chapters.

The GM percentage of RUR soya in all four CRMs was analysed by two rt-PCR techniques and the data obtained were corrected for the DNA mass difference found by the modified Ogur and Rosen method. The first method is targeting a construct-specific part of the genetic modification in this soya and the second one targets an event-specific part of the modification in order to verify the consistency of the certified mass fractions in these CRMs. Genomic DNA was extracted from 200 mg powder samples using the GeneSpin genomic DNA extraction method (Eurofins GeneScan GmbH, Freiburg, DE). Real-time PCR measures copy numbers of the targeted DNA sequences but is calibrated with RUR soya nominal 100 % GM powder. Genomic DNA extracted from the GM powder is diluted in water and analysed by rt-PCR. The absolute GM percentage in a sample was furthermore related to the percentage of the soya-specific lectin reference gene calculated from a calibration curve of the same RUR soya DNA diluted in water. The GM quantity is therefore expressed as a GM percentage. The efficiency of the amplification was determined from the slope of the regression line between the calibrants' percentages and the obtained  $C_t$ -values; for all standard curves, the efficiency was within the limits of the rt-PCR control chart. The results of the GM quantification for the four CRMs are shown in Table 5 and Table 6. The construct-specific method (Table 5) was used to allow comparison of the results obtained from the new batch produced ERM-BF410k and the previous one (ERM-BF410). The event-specific method (Table 6) was used for certification of the present ERM-BF410k (assessment of homogeneity, stability and characterisation).

The quantification of the GM percentage in the powders by rt-PCR confirmed the consistency of the gravimetrically prepared mass fractions of ERM-BF410k.

**Table 5: GM quantification in ERM-BF410k CRMs by construct-specific real-time PCR [e]. Primer and probe sequences can be found in the Annex I. Average of the rt-PCR results obtained by measuring five subsamples from each of five random vials ( $N = 5$ ,**

$n = 5$ ), with each subsample measured in three replicates. Data were corrected for the difference between the DNA content extracted from the GM and non-GM powder.

CRM	RUR soya GM mass fraction <sup>1)</sup> [g/kg]	$U (k = 2)$ [g/kg]
ERM-BF410ak	< 0.09 <sup>2)</sup>	-
ERM-BF410bk	0.80 <sup>3)</sup>	0.09
ERM-BF410dk	10.2	1.0
ERM-BF410gk	101	12

<sup>1)</sup> Real-time PCR measures the GM copy number of the targeted GM DNA sequence in relation to the reference gene, calibrated with DNA extracted from 100 % GM powder and diluted in water.

<sup>2)</sup> The value was below the LOD of the method (0.09 g/kg) for all measurements, each measured in triplicate of the five subsamples from five random vials ( $N = 5$ ,  $n = 5$ ), except in the case of three replicates of one measurement.

<sup>3)</sup> One triplicate of one measurement was excluded as one of the  $C_t$  values was out of the range of the calibration curve. The measurement results from 2 vials were excluded as they generated data below the LOD. For the other measurements, the measured value was above the LOD of the method (0.09 g/kg), each measured in triplicate for five subsamples from three random vials ( $N = 3$ ,  $n = 5$ ).

**Table 6: GM quantification in ERM-BF410k CRMs by event-specific real-time PCR [a]. Primer and probe sequences can be found in the Annex I. Average of the rt-PCR results obtained by measuring five subsamples from each of five random vials ( $N = 5$ ,  $n = 5$ ), with each subsample measured in three replicates. Data were corrected for the difference between the DNA content extracted from the GM and non-GM powder.**

CRM	RUR soya GM mass fraction <sup>1)</sup> [g/kg]	$U (k = 2)$ [g/kg]
ERM-BF410ak	< 0.7 <sup>2)</sup>	-
ERM-BF410bk	0.93 <sup>3)</sup>	0.13
ERM-BF410dk	8.87	1.23
ERM-BF410gk	85.9	8.7

<sup>1)</sup> Real-time PCR measures the GM copy number of the targeted GM DNA sequence in relation to the reference gene, calibrated with DNA extracted from 100 % GM powder and diluted in water.

<sup>2)</sup> The value was below the LOD of the method (0.7 g/kg) for all five subsamples from five random vials ( $N = 5$ ,  $n = 5$ ) of which each was measured in three replicates.

<sup>3)</sup> The measured value was above the LOD of the method (0.7 g/kg) for all measurements, each measured in triplicate for five subsamples from five random vials ( $N = 5$ ,  $n = 5$ ). Two measurements were excluded as some of the  $C_t$  values from the replicates were outside the range of the calibration curve.

## 4 Homogeneity

In order to ensure that the CRMs are sufficiently homogeneous, two strategies were followed: validation of the mixing procedure and control of the homogeneity of the produced mixtures. The adequacy of the dry-mixing technology for the preparation of soya mixtures with different GM mass fractions has been shown before using soya materials processed in the same way as described for ERM-BF410 [3]. Here we only report on the results of a homogeneity study performed on each of the three GM soya mixtures. Additionally, the recommended minimum sample intake is discussed.

### 4.1 Homogeneity study

The homogeneity of ERM-BF410k with respect to the event RUR soya mass fraction was investigated by rt-PCR. Fifteen vials per level, selected according to a random stratified procedure were used. The samples were analysed three times and in random order using a sample intake of 200 mg powder ( $N = 15$ ,  $n = 3$ ). Grubbs tests were performed to detect outlying individual results as well as outlying vial averages. For ERM-BF410bk one outlier was detected for one sample mean of the three replicates measured. For ERM-BF410dk one outlier was detected for one measurement. The outliers were not removed from the data sets as no technical reason for exclusion could be found. For ERM-BF410gk no outliers were detected.

Regression analyses were used to evaluate potential drifts in results related to the analysis sequence or to the filling sequence. No significant trends were observed in the results for ERM-BF410bk and ERM-BF410gk using a 95 % confidence level. For ERM-BF410dk a significant trend at 95 % and 99 % confidence level was observed when looking at the analytical sequence. For the sample means, the trend proved to be significant at 95 % but not at 99 % confidence level. However, these contributions to the between vial heterogeneity were negligible as compared to the maximum hidden heterogeneity.

It was furthermore checked whether the data followed a normal or unimodal distribution using normal probability plots and histograms, respectively. The individual data and the vial averages for all three CRMs appeared to be normally distributed.

ANOVA statistics were used to calculate the relative between vial standard deviation ( $s_{bb, rel}$ ) and the maximum relative standard uncertainty related to the inhomogeneity that can be hidden by the method repeatability ( $u_{bb, rel}^*$ ), using the formulas [10]:

$$s_{bb, rel} = \sqrt{\frac{MS_{bb} - MS_{wb}}{n}} \quad u_{bb, rel}^* = \sqrt{\frac{MS_{wb}}{n}} \cdot \sqrt{\frac{2}{df_{wb}}}$$

( $MS_{bb}$  = mean sum of squares between vials;  $MS_{wb}$  = mean sum of squares within vials;  $n$  = number of replicates;  $df_{wb}$  = degrees of freedom within vials)

Both values were converted into relative uncertainties and were expressed in percentage (Table 7). The larger of the two values was included into the calculation of the overall uncertainty of the certified values (Section 7.2).



**Table 7: Relative standard uncertainties due to heterogeneity between vials of dry-mixed RUR soya CRMs, as analysed by event-specific rt-PCR [a]**

CRM	Number of samples analysed	Relative between vial heterogeneity ( $s_{bb,rel}$ ) [%]	Relative maximum hidden heterogeneity ( $u^*_{bb,rel}$ ) [%]
ERM-BF410bk	$N = 15, n = 3$	- <sup>1)</sup>	5.3
ERM-BF410dk	$N = 15, n = 3$	- <sup>1)</sup>	3.5
ERM-BF410gk	$N = 15, n = 3$	1.2	2.2

<sup>1)</sup> As  $MS_{bb}$  was smaller than  $MS_{wb}$ ,  $s_{bb,rel}$  could not be calculated.

The materials can therefore be considered to be homogeneous, with a maximum hidden heterogeneity of 5.3 %.

## 4.2 Minimum sample intake for analysis

Many commonly employed DNA extraction methods for plant powders recommend the use of 100 or 200 mg of powder as sample intake. A mass of 200 mg powder was employed throughout this certification project for DNA extraction by either CTAB in combination with Genomic tip20/G or the GeneSpin method. The assumption that a subsample of this size is representative for the whole batch was investigated as follows.

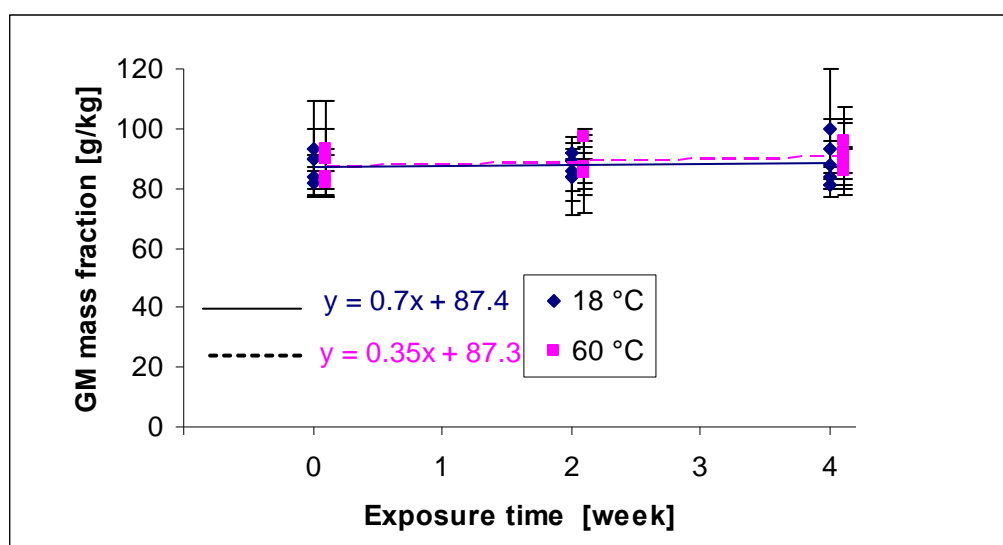
The mass density of the non-GM soya seed powder was determined by so-called tap-density measurements using the procedure described in [11]. Taking into account the mass density determined for the non-GM soya (0.54 g/mL) and the GM soya (0.57 g/mL) and the particle size distribution (average particle size of approximately 171  $\mu\text{m}$  as equivalent volume diameter), it was calculated that a 200 mg sample roughly contains  $1.4 \times 10^5$  seed tissue powder particles.

As a general rule, it is advised to use sample intakes not smaller than 200 mg.

## 5 Stability

### 5.1 Short-term stability

In order to assess whether special care must be taken during transportation, a short-term stability of the dried soya seed powder, produced in the same way as ERM-BF425 (356043 soya), was investigated using an isochronous approach [12]. ERM-BF410gk was chosen for this study as it contained the highest GM mass fractions of the Roundup Ready event (nominal 100 g/kg). Five randomly selected bottles were stored at each of the temperatures 18 °C and 60 °C during 2 and 4 weeks, and three subsamples from each bottle were analysed ( $N = 5$ ,  $n = 3$ ). The same number of reference samples was likewise stored at -70 °C. Genomic DNA was extracted from the samples by the GeneSpin method, the extractable DNA content was determined by the PicoGreen® method and visualised by gel electrophoresis. No substantial DNA degradation was seen in any of the samples. Each DNA extract was analysed in triplicate by event-specific rt-PCR to reveal changes in GM quantification (Figure 3). Scrutinising the data obtained, one outlier was detected at 60 °C (at 95 % confidence level) by Grubbs tests. As no technical reason could be given to exclude this data, it was kept in the statistical analysis. Regression analysis was performed for each of the storage temperatures to reveal any trend in GM quantity in relation to the time of storage. A  $t$ -test showed absence of trend after storage at 18 °C and 60 °C for a time period of 4 weeks (95 % confidence level). Therefore, it was concluded that the possibility of degradation during dispatch at ambient temperature (always shorter than 4 weeks) is negligible for ERM-BF410gk. This statement can reasonably be extended to ERM-BF410ak, bk and dk given the similar composition of the four CRMs.



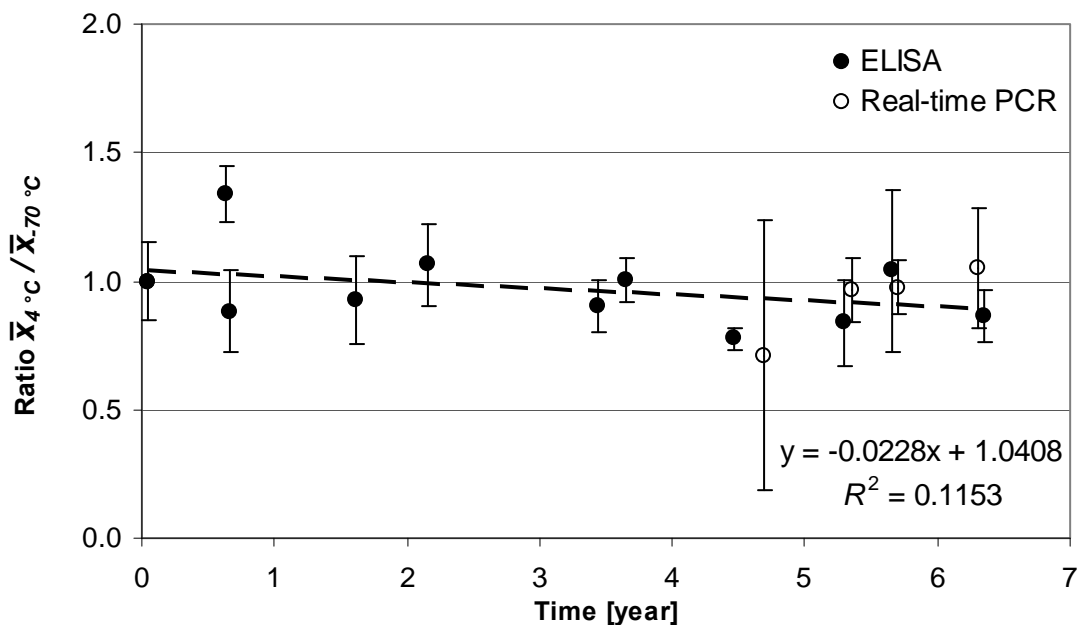
**Figure 3: Short-term stability of ERM-BF410gk stored at 18 °C (solid line) and 60 °C (dashed line) for 2 and 4 weeks and analysed by event-specific rt-PCR. The exposure time 0 week refers to the results obtained for samples stored at the -70 °C reference temperature during the 4-weeks study. The bars indicate the interval  $\bar{x} \pm s$  for  $N = 5$ ;  $n = 3$ .**

It was concluded that the uncertainty due to degradation during dispatch is negligible for all four CRMs. ERM-BF410k can be shipped under ambient conditions.

## 5.2 Long-term stability

From post-certification monitoring analysis, the long-term stability of soya CRMs (i.e. ERM-BF410) during storage has been monitored at IRMM for more than 6 years, using ELISA and/or event-specific rt-PCR methods (Figure 4, based on own unpublished results). Although visually there seems to be a downward trend in the stability data over the time period investigated, statistical evaluation did not reveal this to be significant ( $p = 0.15$ ,  $t$ -test, 95 % confidence level). The uncertainty contribution for one year storage ( $u_{\text{ITS,rel}}$ ) [14], calculated from the available long-term stability data, was approximately 1.9 % of the certified value.

It can be concluded that the storage conditions at IRMM are suited for the long-term storage of soya CRMs. As for all GMO CRMs, a post-certification monitoring scheme is put in place in order to continue monitoring the stability of ERM-BF410k.



**Figure 4: Long-term stability of dried soya seed powder (ERM-BF410) stored at 4 °C for various time periods, based on ELISA (●) and rt-PCR (○) measurements. The values are expressed as the ratio between the GM mass fraction ratio in samples stored at 4 °C and that in samples stored for the same time period at the reference temperature (- 70 °C), with the bars indicating the expanded uncertainty interval  $\pm U$  ( $k = 2$ ). Each bullet corresponds to the average of 2 to 9 measurements. The dashed line is the regression line generated on the basis of all data points. The monitoring was performed with the nominal 1 % GM soya powder. The stability results can be reasonably extended to other GM mass fractions of soya powder.**

## 6 Characterisation

The four CRMs of ERM-BF410k are soya powder CRMs processed from pure non-GM and pure GM base materials. While ERM-BF410ak is prepared from the pure non-GM blank material, ERM-BF410bk, dk, gk are gravimetrically produced mixtures of the pure non-GM and GM powders. The certified value is based on the GM mass fraction of dry-mixed GM and non-GM powder, corrected for their water mass fractions, and taking into account the powder's purity with regard to the GTS 40-3-2 event. Assuming that the purity of the non-GM powder is 100 %, which was supported by the data (Section 3.1), the GM mass fractions can be calculated according to the following formulas:

$$\text{GM mass fraction [g/kg]} = \frac{m_{\text{GM,anhyd}} [\text{g}] \times p_{\text{GM}} [\text{g/g}]}{m_{\text{GM,anhyd}} [\text{g}] + m_{\text{nonGM,anhyd}} [\text{g}]} \times 1000$$

$$m_{\text{GM,anhyd}} [\text{g}] = m_{\text{GM}} [\text{g}] \times (1 - \text{WMF}_{\text{GM}} [\text{g/g}])$$

$$m_{\text{nonGM,anhyd}} [\text{g}] = m_{\text{nonGM}} [\text{g}] \times (1 - \text{WMF}_{\text{nonGM}} [\text{g/g}])$$

(anhyd = anhydrous;  $p_{\text{GM}}$  = purity of the GM powder used for the dilution; WMF = water mass fraction)

For the purity of the GM base material the genetic identity of randomly selected seeds has been checked (Section 3.1). No evidence of the occurrence of non-GM seeds among the GM seeds was found. Based on a statistical analysis of the distribution of the probability to find a negative seed in the GM base material, it could be concluded that the purity was higher than 94 % (95 % confidence level). For the calculation of the certified value, a GM purity of the seed batch of 100 % was used, based on the actual number of positive seeds detected per total number of seeds analysed (46 out of 46 seeds tested, 4 seeds did not germinate).

## 7 Certified values and uncertainty budgets

### 7.1 Certified value

The certified value is based on the masses of dried powder of GM seeds and non-genetically modified seeds used in the gravimetric preparation. The masses of the powders are corrected for their respective water mass fractions and their estimated GTS 40-3-2 soya purity (see Table 2 in Section 3.3).

The seed batches used for the processing of these powders were thoroughly checked for any impurity. No indication of the presence of the GTS 40-3-2 soya was found in the non-GM seed lot by rt-PCR, supporting the quality control results of the company supplying the seeds (Section 3.1).

For the GM seeds, no indication was found for the absence of the GTS 40-3-2 soya in any of the individual seedlings raised from the GM seed lot when measured by event-specific rt-PCR (Section 3.1). Processing control of ERM-BF410bk, dk, gk showed a similar GM mass fraction as the certified value (Table 5 and 6).

As a consequence of the purity of the seed batches used, the CRMs based on gravimetric mixtures of GM and non-GM powders could be certified for values equal to the intended nominal GM mass fractions (Table 2 and 8).

### 7.2 Uncertainty budget

Controlled processing techniques in combination with purity controls of the GM and non-GM seeds and the derived powder base materials allowed certifying the GM mass fractions in the CRMs with rather small uncertainties.

The combined standard uncertainty of the certified value comprises contributions from the between-vial inhomogeneity at the recommended sample intake of 200 mg ( $u_{bb}$ ), the long-term stability of the material ( $u_{lts}$ , calculated for 12 months) and the characterisation of the materials ( $u_{char}$ ). The  $u_{char}$  includes uncertainties related to the weighing procedure, the determination of the water mass fraction in the powders, and the analysis of the purity of non-GM and GM base materials (Table 8). To calculate the expanded uncertainty corresponding to a 95 % level of confidence a coverage factor of 2 was used [15].

For the blank material, the LOD of the method was used to describe the 95 % confidence interval on the certified value ( $< 0.7$  g/kg). This is supported by the high purity of the (non-GM) material and the absence of any mixing step; calculating the  $U_{CRM}$  for the blank material on the basis of the only quantifiable standard uncertainty ( $u_{char,3}$ ) resulted in a value of 0.2021 g/kg, which is below the certified  $< 0.7$  g/kg.

**Table 8: Uncertainty budgets for the mass fractions of GTS 40-3-2 soya in ERM-410k**

ERM code	Certified value [g/kg]	Standard uncertainty contribution [g/kg]						Expanded uncertainty $U_{CRM} (k = 2)$ [g/kg]
		$u_{bb}$ <sub>2)</sub>	$u_{lts}$ <sub>3)</sub>	$u_{char,1}$ <sub>4)</sub>	$u_{char,2}$ <sub>5)</sub>	$u_{char,3}$ <sub>6)</sub>	$u_{char,4}$ <sub>7)</sub>	
<b>BF410ak</b>	<b>&lt; 0.7</b> <sup>1)</sup>	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	<b>n.a.</b>
<b>BF410bk</b>	<b>1.0</b>	0.0530	0.0187	0.0018	0.00002	0.2021	0.0188	<b>0.5</b>
<b>BF410dk</b>	<b>10.0</b>	0.3500	0.1870	0.0149	0.00015	0.2021	0.1879	<b>1.0</b>
<b>BF410gk</b>	<b>100</b>	2.2002	1.8704	0.1053	0.00170	0.2021	1.8797	<b>7</b>

<sup>1)</sup> With a 95 % probability, the certified value is below this level.

<sup>2)</sup> Standard uncertainty contribution resulting from the homogeneity assessment (Table 7).

<sup>3)</sup> Standard uncertainty resulting from the stability of dried soya seed powders during storage, extrapolated to 12 months.

<sup>4)</sup> Standard uncertainty of the mass determination based on the uncertainty of the balance and the number of weighing steps required.

<sup>5)</sup> Standard uncertainty of the water mass fraction determination by volumetric KFT, based on the highest standard uncertainty of the method (14.5 % for the measurements of the nominal 10 g/kg GM powder) and the highest water mass fraction found in any of the powders used for mixing (11.7 g/kg for the non-GM powder).

<sup>6)</sup> Standard uncertainty introduced by the purity of the non-GM base material (LOD = 0.7 g/kg), based on the half-width of the interval between 0 and 0.7 g/kg, divided by the square root of 3 (rectangular distribution).

<sup>7)</sup> Standard uncertainty introduced by the purity of the GM base material (> 94 %), based on the half-width of the interval between 94 % and 100 % divided by the square root of 3 (rectangular distribution).

## 8 Metrological traceability

The ERM-BF410k series is composed of four reference materials certified for the mass fraction of event GTS 40-3-2 soya seed powder. The certified values are based on gravimetric dry-mixing of event GTS 40-3-2 soya seed powder with non-modified soya seed powder. The respective certified values are traceable to the SI. The traceability chain is based on the use of calibrated balances and a thorough control of the weighing procedure. The purity of the used seeds has been taken into account when calculating the certified value.

The user of the certified reference material should, however, bear in mind that the values for the GM percentage or copy number measured by rt-PCR could potentially differ from the certified GM mass fraction as a result of different DNA mass extracted from a given sample intake of GM and non-GM powders. Depending on the variety composition of the unknown sample measured in connection with ERM-BF410k, rt-PCR measurement results of ERM-BF410k and the unknown sample may differ (average  $\pm U$ ) up to  $(18 \pm 8) \%$ . This difference may arise from the DNA mass difference between the sample intake of non-GM and GM powders used for the production of ERM-BF410k and may depend also on the DNA extraction method selected. The user should bear in mind that DNA mass differences may additionally arise from the unknown sample depending on the composition of this sample and that these two effects may be additive (Section 3.6).

## **9 Intended use and instructions for use**

ERM-BF410ak, bk, dk, gk are intended for use as quality control materials or calibrants in DNA- or protein-based methods for the detection of genetically modified material in food and feed. However, one has to be careful to draw quantitative conclusions (in gene copy numbers, for instance) from measurements of unknown samples as DNA- and/or protein-based GM quantification may vary with the particular matrix and the soya variety tested [9].

The recommended minimum sample intake is 200 mg.

The materials are hygroscopic. Vials should be stored dry and in the dark at maximum 4 °C. The user is advised to close vials immediately after taking a sample for analysis.



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## **ANNEX 1**

### **Event-specific real-time PCR for soya 40-3-2 [a]**

#### **Endogenous target / lectine**

Primer\*:

TM-Lectin-F: 5'-tcc acc ccc atc cac att t-3'

TM-Lectin-R: 5'-ggc ata gaa ggt gaa gtt gaa gga-3'

Probe:

Lectin-FAM: 5'-(FAM)-aac cgg tag cgt tgc cag ctt cg-(TAMRA)-3'

**OR**

Primer\*:

*lecF*: 5'-cca gct tcg ccg ctt cct tct-3'

*lecR*: 5'-gaa ggc aag ccc atc tgc aag cc-3'

Probe:

*lec P*: 5'-(FAM)-ctt cac ctt cta tgc ccc tga cac-(TAMRA)-3'

\*It was experimentally demonstrated that the use of both lectin-specific primers/probe in combination with transgenic target-specific primers/probe generated equivalent data when performing real-time PCR measurements.

#### **Transgenic target / *epsps***

Primer:

40-3-2 AF: 5'-ttc att caa aat aag atc ata cat aca ggt t-3'

40-3-2 AR: 5'-ggc att tgt agg agc cac ctt-3'

Probe:

40-3-2 AP: 5'-(FAM)-cct ttt cca ttt ggg-(MGBNFQ)-3'

### **Epsps specific real time PCR [b]**

#### **Endogenous target / lectine**

Primer:

TM-Lectin-F: 5'-tcc acc ccc atc cac att t-3'

TM-Lectin-R: 5'-ggc ata gaa ggt gaa gtt gaa gga-3'

Probe:

Lectin-FAM: 5'-(FAM)-aac cgg tag cgt tgc cag ctt cg-(TAMRA)-3'

#### **Transgenic target / *epsps***

Primer:

RRS-F: 5'-gcc atg ttg tta att tgt gcc at-3'

RRS-R: 5'-gaa gtt cat ttc att tgg aga gga c-3'

Probe:

RRS-FAM: 5'-(FAM)-ctt gaa aga tct gct aga gtc agc ttg tca gcg-(TAMRA)- 3'

### **CaMV 35S real-time PCR [c]**

#### **Endogenous target / lectine**

Primer:

TM-Lectin-F: 5'-tcc acc ccc atc cac att t-3'

TM-Lectin-R: 5'-ggc ata gaa ggt gaa gtt gaa gga-3'

Probe:

Lectin-FAM: 5'-(FAM)-aac cgg tag cgt tgc cag ctt cg-(TAMRA)-3'

#### **Transgenic target / CaMV 35S**

Primer:

TM-35S-1: 5'-gcc tct gcc gac agt ggt-3'

TM-35S-2: 5'-aag acg tgg ttg gaa cgt ctt c-3'

Probe:

35S-FAM: 5'-(FAM)-caa aga tgg acc ccc acc cac g-(TAMRA)-3'

#### **CaMV NOS real-time PCR [d]**

##### **Endogenous target / lectine**

Primer:

TM-Lectin-F: 5'-tcc acc ccc atc cac att t-3'

TM-Lectin-R: 5'-ggc ata gaa ggt gaa gtt gaa gga-3'

Probe:

Lectin-FAM: 5'-(FAM)-aac cgg tag cgt tgc cag ctt cg-(TAMRA)-3'

##### **Transgenic target / CaMV NOS**

Primer:

Nost5'F: 5'-ttg gca ata aag ttt ctt aag att gaa t-3'

Nost5'R: 5'-aca tgc tta acg taa ttc aac aga aat t -3'

Probe:

NOS-FAM: 5'-(FAM)-ctg ttg ccg gtc ttg cga tga tta tca t-(TAMRA)-3'

#### **Construct-specific real-time PCR for soya 40-3-2 [e]**

##### **Endogenous target / lectine**

Primer:

*lecF*: 5'-cca gct tcg ccg ctt cct tct-3'

*lecR*: 5'-gaa ggc aag ccc atc tgc aag cc-3'

Probe:

*lec P*: 5'-(FAM)-ctt cac ctt cta tgc ccc tga cac-(TAMRA)-3'

##### **Transgenic target / 40-3-2**

Primer:

RRS-F: 5'-gcc atg ttg tta att tgt gcc at-3'

RRS-R: 5'-gaa gtt cat ttc att tgg aga gga c-3'

Probe:

RRS-FAM: 5'-(FAM)-ctt gaa aga tct gct aga gtc agc ttg tca gcg-(TAMRA)-3'

**EUR 23504 EN – Joint Research Centre – Institute for Reference Materials and Measurements**

Title: Certification of reference materials of soya seed powder with different mass fractions of genetically modified Roundup Ready<sup>®</sup> soya, ERM<sup>®</sup>-BF410k (ERM<sup>®</sup>-BF410ak, ERM<sup>®</sup>-BF410bk, ERM<sup>®</sup>-BF410dk, ERM<sup>®</sup>-BF410gk)

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**Abstract**

This report describes the processing and certification of four soya seed powder Certified Reference Materials (CRMs) containing different mass fractions of genetically modified (GM) GTS 40-3-2 (Roundup Ready) soya (ERM-BF410ak, bk, dk, gk). The materials were processed and certified in 2007/2008 by the European Commission, Joint Research Centre, Institute for Reference Materials and Measurements (IRMM, Geel, Belgium), according to the principles of ISO Guide 34.

Homozygous seeds of GM GTS 40-3-2 soya and of a comparable non-GM soya variety were dried and ground to GM and non-GM base powders. A non-GM pure material and three gravimetric mixtures of non-GM and GM soya powder (containing respectively 1.0, 10.0 and 100 g/kg GM soya) were prepared by dry-mixing. The remaining > 500 µm particles were then sieved out from each of these powders, milled a second time, and added back to the main fraction. The certified values of these CRMs were calculated from the gravimetric preparations, taking into account the GM purity of the base materials and their water mass fraction. The certified values were confirmed by event-specific real-time PCR as independent verification method (measurements within the scope of accreditation to ISO/IEC 17025).

The four CRMs belonging to the ERM-BF410k set replace the beforehand produced set ERM-BF410, which is close to be sold out. ERM-BF410k is certified to contain the following GTS 40-3-2 soya mass fractions:

CRM	Certified value: GTS 40-3-2 soya mass fraction <sup>1)</sup> [g/kg]	Uncertainty <sup>2)</sup> [g/kg]
ERM-BF410ak	< 0.7 <sup>3)</sup>	-
ERM-BF410bk	1.0	0.5
ERM-BF410dk	10.0	1.0
ERM-BF410gk	100	7

<sup>1)</sup> The certified value is based on the mass fraction of GTS 40-3-2 soya seed powder mixed in non-genetically modified soya seed powder and taking into account their respective GTS 40-3-2 soya purity and their water mass fraction. The certified value is traceable to the SI.

<sup>2)</sup> The certified uncertainty is the expanded uncertainty (*U*) estimated in accordance with the Guide to the Expression of Uncertainty in Measurement with a coverage factor *k* = 2, corresponding to a level of confidence of about 95 %.

<sup>3)</sup> With a 95 % probability, the value of the material is below this level.

The CRMs are intended to be used for quality control or calibration of methods for the quantification of the GTS 40-3-2 soya mass fraction in food and feed. The CRMs are available in glass vials containing 1 g of dried soya powder closed under argon atmosphere. The minimum amount of sample to be used per analysis is 200 mg.

The four CRMs have been accepted as European Reference Material (ERM<sup>®</sup>) after peer evaluation by the partners of the European Reference Materials consortium.

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